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A high-throughput method for screening of rapamycin-producing strains of *Streptomyces hygroscopicus* by cultivation in 96-well microtiter plates

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Abstract

A novel high-throughput cultivation method was developed to rapidly screen large numbers of rapamycin-producing mutants of *Streptomyces hygroscopicus* by duplicate culturing of isolates on the surfaces of agar-solidified 96 wells in microtiter plates. One copy of the cultures was used for the rapamycin bioassay and the other identical copy, representing potentially high yielding strains, was preserved for further study. By integrating 96-well solid cultivation and the bioassay, we screened more than 7000 isolates and found 10 high-yielding strains. From these, one mutant produced 420 μg rapamycin/ml, which was double the yield of parent strain used in the submerged fermentation process.

Introduction

Rapamycin is an antifungal antibiotic produced by *Streptomyces hygroscopicus* (Sehgal *et al.* 1975, Vezina *et al.* 1975). It also has potent immunosuppressive activity by inhibiting T-cell activation and proliferation (Biere *et al.* 1990, Dumont *et al.* 1990). It has been approved in United States for use in combination with cyclosporine and corticosteroids for various treatments (Kahan *et al.* 1991, Kaplan *et al.* 1998). Compared with its competitive agents, the productivity of rapamycin in *Streptomyces hygroscopicus* is very low, being only about 200–300 mg/l (Kojima *et al.* 1995, Lee *et al.* 1997, Chen *et al.* 1999). Further improvement in productivity of this industrial microorganism is imperative for commercial success.

For the past decade, new technologies have been developed to improve productivity of industrial strain, such as combinatorial biosynthesis and genome shuffling (Zhang *et al.* 2002, Donadio *et al.* 2003, Petri *et al.* 2004). However, traditional mutation is still used to improve industrial strains. A search for a high-yielding strain depends largely on the total number of mutants that can be screened after mutagenesis (Vinci *et al.* 1991). Even with genome shuffling strategy, positive mutants must be screened out from a large number of isolates obtained by using recursive protoplast fusion (Zhang *et al.* 2002). A variety of screening methods have been developed targeting different types of microorganisms (Filtenborg *et al.* 1983, Smedsgaard *et al.* 1997, Bragulat *et al.* 2001). For antibiotic-producing strains, the "agar plug" method was widely used in 1980s, and many modified versions were developed for preliminary screening with high efficiency (Du Toit *et al.* 2000, Kumar *et al.* 2000).

The aim of this work was to develop a high-throughput screening (HTS) technique to isolate the high rapamycin-producing strains from a large number of mutants after nitrosoguanidine treatment of *Streptomyces hygroscopicus*. The combination of 96-well microtiter-plates cultivation

technique and agar-plug method was used to make the screening procedure more efficient. The productivity by rapamycin by the isolated strains was then examined by submerged fermentation.

Materials and methods

Microorganisms and mutation

Streptomyces hygroscopicus ZX-S–21 was isolated after mutation of *S. hygroscopicus* ATCC 29253 and preserved in our laboratory. This low-level rapamycin producer was grown at 28 °C for 20 days on solid medium (oat meal 20 g/l, agar 20 g/l, pH 7.0). Spores were harvested in 15 ml sterile 0.85% saline containing 0.1% Tween 80 and 2 ml of this suspension was mixed with 0.2 ml of 5 mg nitrosoguanidine (NTG)/ml, and killing ratio of ca. 90% was obtained by shaking the mixture for 1 h at 150 rpm. The spore suspension, containing 10–1000 c.f.u., was prepared. For each Petri dish, 0.2 ml of the spore suspension was spread onto the surface of SYPC medium (soluble starch 10 g/l, yeast extract 6 g/l, peptone 6 g/l, Casamino acids 1.5 g/l, $MgSO_4$ 0.5 g/l, K_2HPO_4 1.0 g/l, pH 6.3–6.8). After cultivation for 10 days at 28 °C, separate colonies appeared with a large number of spores.

Candida albicans (ATCC 11651) was used in the bioassay of rapamycin. It was grown at 28 °C for 2 days on an agar slant (glucose 20 g/l, yeast extract 10 g/l, peptone 20 g/l, agar 20 g/l, pH7.0).

Cultivation of isolates on 96-well agar array and extract of rapamycin

Each well in aseptic 96-well microtiter plates was filled with 0.2 ml sterile SYPC medium containing 1.5% agar. After solidification, the spores from one colony in the Petri dish were transferred into one corresponding well of the duplicate microtiter plates (A and B). After every well was inoculated, the 96-well plates were put into a 180 × 180 mm Petri dish in which 100 ml sterile water and 25 pieces of glass beads were added to prevent the agar column from drying. The dishes were incubated at 28 °C for 10 days in a biochemical oxygen demand (BOD) incubator. Then, each medium column in one 96-well plate (A) was plugged out totally and transferred into a 1.5 ml Eppendorf tube in which 0.5 ml methanol was pre-added. After 6 h shaking at 30 °C, methanol extracts were centrifuged for 15 min at 5000 *g*, and the supernatant was then used for bioassay. Another 96-well plate (B) was stored in refrigerator at 4–6 °C. If the strain in one well of plate (A) showed high rapamycin productivity by bioassay, the strain in the corresponding well of plate (B) will be further validated by submerged fermentation. Control plugs were also prepared with *S. hygroscopicus ZX-S–21* and harvested using the same procedure.

Bioassay method for rapamycin

Rapamycin production was assayed using a paper disc-agar diffusion method using *Candida albicans* ATCC 11651 as the indicator (Kojima *et al.* 1995, Lee *et al.* 1997). The agar medium dispensed in a 90 × 90 mm Petri dish composed of two separate layers. First, 15 ml test medium (glucose 5 g/l, peptone 2 g/l, agar 20 g/l) formed a base layer in the Petri dish then, after solidification, 0.1 ml suspension of *C. albicans* mixed with 5 ml of sterile saline containing 0.8% agar at 40–50 °C was immediately poured onto the base layer to constitute the upper one. Then 12 pieces of 6 mm diam. paper disk were placed onto each dish, and 6 μl methanol extract from the agar plug of plate (A) was transferred onto 10 paper disks respectively. In the remaining two paper disks, one containing 6 μl pure methanol was used as negative standard, the another containing 6 μl methanol extract from the parent strain was used as positive standard. In order to facilitate rapamycin in the methanol extract diffusing into the medium of Petri dish, the plates were stored at 4 °C for 12 h, then incubated for 2 days at 30 °C. The inhibitory zone was observed and the diameter was measured. The larger the diameter of inhibitory zone is, the higher the concentration of rapamycin in methanol extract will be; therefore, the strain with the largest inhibitory zone can be easily picked out and the corresponding strain in plate (B) will be further examined by shake-flask submerged fermentation. The detailed procedure of this high-throughput screening technique is illustrated in Figure 1.

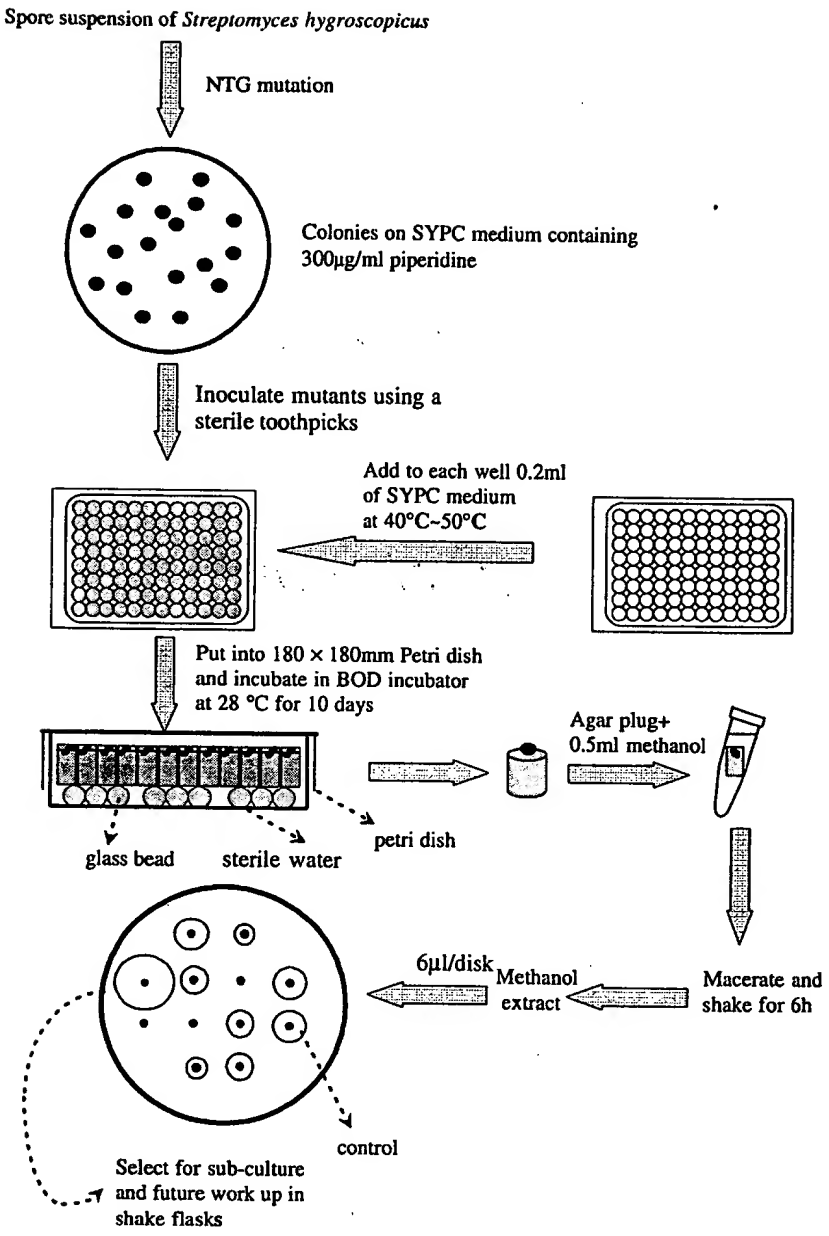

Fig. 1. An integrated process with a modified agar plug method and *Candida albicans* bioassay.

Shake flask screening for high-yielding isolates

Spores in plate (B) picked out according to bioassay were inoculated into a 250 ml Erlenmeyer flask containing 30 ml seed medium (soluble starch 10 g/l, yeast extract 6 g/l, peptone 6 g/l, Casamino acids 1.5 g/l, $MgSO_4$ 0.5 g/l, K_2HPO_4 1 g/l, pH 7.0). Incubation was conducted at

28 °C for 60 h on a rotary shaker (220 rpm), and 3 ml of the resulting culture broth was transferred into a 250 ml Erlenmeyer flask containing 30 ml medium. Fermentation was continued for 5 days at 28 °C on a rotary shaker (240 rpm). Then 2 ml of fermentation broth was centrifuged for 15 min at 4000 *g*. The cell pellet was mixed with the same volume of methanol and shaken intensively for 4 h. After centrifugation at 4000 *g* for 15 min, the supernatant was subjected to HPLC analysis to determine rapamycin concentration. An Agilent 1100 series HPLC system equipped with a Hypersil BDS C_{18} reversed-phase analytical column (250 × 4.6 mm I.D., 5 μm particle) was used. The mobile phase consisted of methanol/water (78:22, v/v) at 1 ml/min and the eluate was monitored at 277 nm. Before analysis, the samples needed to be diluted properly, and rapamycin in the sample was estimated using an internal standard.

Results and discussion

Determination of cultivation time of isolates in 96-well microtiter plate

The 96-well microtiter plate is able to culture many mutants on the surface of agar-solidified medium simultaneously with uniform conditions. The mutant of *S. hygroscopicus* grew well on the plate for more than 20 days. In order to determine the optimal cultivation duration for rapamycin production, the spores of *S. hygroscopicus ZX-S–21* were used to inoculate three 96-well microtiter plates in parallel. At intervals, three samples were taken out from 96-well plates and extracted with methanol. The productivity of rapamycin was determined by the diameter of inhibitory zones against *C. albicans* ATCC 11651. The results were shown in Figure 2. The discs with pure methanol, used as a negative control, showed no inhibitory zone. The inhibitory zones with *S. hygroscopicus ZX-S–21* were round with sharp edge. After cultivation of *S. hygroscopicus* for four days, rapamycin started to be synthesized and the inhibitory zone size was expanded quickly with time and stopped to increase until the 10th day. Further extending of culture time did not increase the inhibitory zone size and even decreased after 14th day. Based on above observations, in the following experiments, the cultivation time in the 96-well microtiter plate was fixed for 10 days.

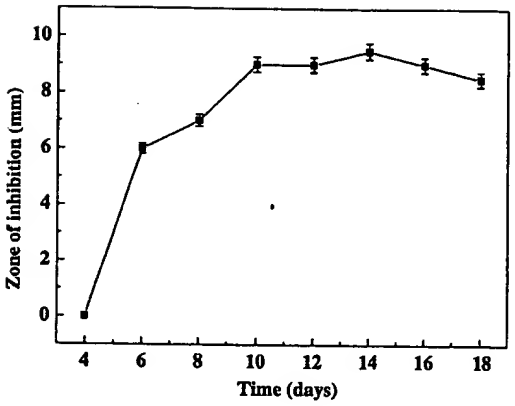

Fig. 2. The relationship between the diameter of inhibition zone and the incubation time of *S. hygroscopicus* on the surface of 96 solidified agar wells. All the experiments were carried out in triplicate, and values are the average of three independent determinations.

Solvent extraction of rapamycin from the agar plug

Rapamycin is an intracellular product. To examine its inhibitory zone, it is necessary to extract rapamycin from mycelium of *S. hygroscopicus* grown on the agar plugs in the 96-well microtiter plate. Various organic solvents were evaluated and methanol showed the highest efficiency. The time course of methanol extraction was shown in Figure 3. Complete extraction of rapamycin needed 6–8 h. Efficient extraction of rapamycin by methanol from *S. hygroscopicus* pellets takes 2 h (Lee *et al.* 1997). The extension of extraction time is probably caused by the mass transfer resistance when the mycelia are partially wrapped by the agar. In the following experiments, methanol extraction for 6 h was adopted for further bioassay.

Development of HTS method for bioassay of rapamycin

Although *C. albicans* ATCC 11651, used as test organism, is sensitive to rapamycin, the sensitivity

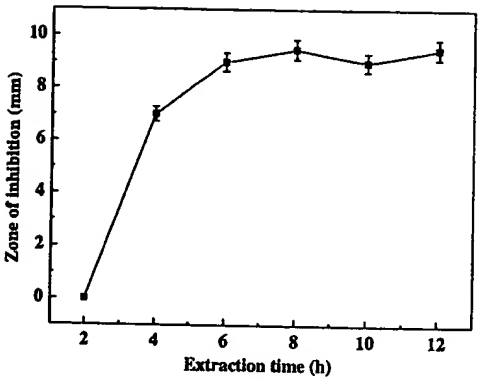

Fig. 3. The relationship between the extract time of rapamycin from an agar plug and the diameter of inhibition zone. All the experiments were carried out in triplicate, and values are the average of three independent determinations.

and consistency of inhibitory zone size is dependent on the growth period. After detailed experimental evaluation, the best results were obtained that *C. albicans* ATCC 11651 in double-layer medium should be incubated for 16–18 h at 28 °C.

After above optimization processes of key operation conditions, one high throughput screening (HTS) procedure with 96-well formats was successfully developed in our laboratory, and was used to screen high rapamycin-producing isolates.

A total of 2108 separate colonies appeared in SYPC medium after treatment of spore suspension of *S. hygroscopicus ZX-S–21* with NTG. Of these, 2008 colonies grew well in 96-well microtiter plates; those with poor or no growth were discarded. By running the HTS procedure, those isolates providing the largest inhibition zones over the original one were selected as a population of positive. The size of inhibitory zones from selected isolates were measured and recorded. The respective duplicate agar plugs (B) were then subcultured to validate rapamycin productivity in shake-flask fermentation. Each isolate was incubated in triple and the rapamycin productivity in each flask was analyzed by HPLC. The final rapamycin concentration was the average of that in three parallel flasks. The results showed that three isolates (N102, N437, N758), which gave the largest sizes of inhibitory zones, were the top-three rapamycin producers in the shake-flask fermentation.

The spores of these three mutants were mixed together and used for next-run mutation and screening. After three-run similar work, 10 high-yielding strains were screened out from more than 7000 mutants. There was an almost linear relationship between inhibitory zone sizes of the 10 high-yielding strains and the corresponding rapamycin production in the shake-flask culture (Figure 4). Strain N5632 was the most productive one from three rounds of the HTS procedure and the rapamycin yield reached 420 mg/l, which is the highest value ever reported in the literature in submerged fermentation and is double that produced by the original ZX-S–21. A 124% improvement of rapamycin productivity was achieved by traditional mutation breeding, however, the highest rapamycin productivity of mutant C14–2 was only 139 mg/l (Cheng *et al.* 2001).

The advantages of the HTS method developed in this work are its high throughput and ease of isolating high producing strains. Within 3 months, three rounds of HTS can be performed and more than 7000 isolates being evaluated. Also expensive and tedious HPLC analysis can be reduced to the minimum. The new HTS procedure is versatile for the screening of high producing strains for antibiotic production and may be applied in the other screening systems after improvement. In this laboratory, the HTS procedure is being applied in the genome shuffling.

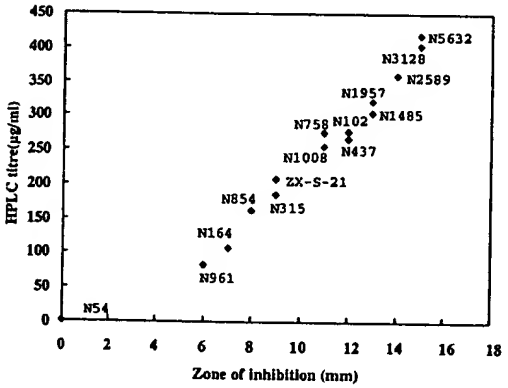

Fig. 4. Zones of inhibition of *C. albicans* against HPLC titre of rapamycin in shake flask. All the experiments were carried out in triplicate, and values are the average of three independent determinations.

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